Accurate Estrogen Receptor Quantification in Patients with Negative and Low-Positive Estrogen-Receptor-Expressing Breast Tumors: Sub-Analyses of Data from Two Clinical Studies

Original Research Article

Accurate Estrogen Receptor Quantification in Patients with Negative and Low-Positive Estrogen-Receptor-Expressing Breast Tumors: Sub-Analyses of Data From Two Clinical Studies

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Summary Slide (4-5 bullet points):

- *Why carry out this study?*
    - Accurate assessment of estrogen receptor (ER) expression is crucial to identify those breast cancer patients who are most likely to benefit from endocrine therapy.
    - Current clinical practice utilizes an ER threshold of ≥ 1% staining by immunohistochemistry (IHC); however, this threshold may not accurately classify patients. Reverse-transcription polymerase chain reaction (RT-PCR) may provide a more precise indication of ER status.
    - The aim of this study was to compare ER expression measured by IHC and the 21-gene Oncotype DX Breast Recurrence Score® assay, a multigene assay that employs RT-PCR-based methodology that has been clinically validated to determine the benefit of chemotherapy in patients with ER-positive (ER+) early stage breast cancer when treated with endocrine therapy.
- *What was learned from the study?*
    - There was a 100% agreement between samples labelled as ER+ (> 10% staining) by IHC and by RT-PCR; however, slightly more than half (56%) of samples classified as ER-low+ by IHC (1–10% staining) were classified as ER-negative by RT-PCR.
    - In patients with low ER expression by IHC, the 21-gene assay provides more precise information than IHC in patients with ER-low+ expression (1–10%), which can be used to determine which patients are likely to benefit from endocrine therapy.

Advances in Therapy

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ABSTRACT

Introduction: Accurate assessment of estrogen receptor (ER) expression is crucial to ensure that patients with early breast cancer are accurately identified for appropriate treatment with endocrine therapy. Reverse-transcription polymerase chain reaction (RT-PCR), compared with immunohistochemistry (IHC), may provide a more precise indication of ER status. Data were pooled and analyzed from two independent, but similarly designed studies that examined ER status by IHC and the 21-gene Recurrence Score that employs RT-PCR-based methodology,

Methods: Tumor tissue from patients with early stage breast cancer where ER status could be determined by both IHC and RT-PCR was included. ER status by IHC staining was defined as ER-negative (< 1%), ER-low+ (1–10%), or ER+ (> 10%). ER Status by RT-PCR was defined as ER-negative (≤ 6.5) or ER+ (> 6.5). Recurrence Score results from the 21-gene assay were reported on a continuous scale from 0 to 100. A sub-analysis examined the association between ER expression (Allred score 2–7) and response to a 14-day pre-surgery pulse with an aromatase inhibitor. A separate sub-analysis examined the association between ER expression and human epidermal growth factor receptor 2 (HER2) expression.

Results: Tumor specimens from 192 patients (aged 25–92 years) were included in the pooled analysis. Correlation between IHC- and RT-PCR-measured ER was strong for IHC-defined ER-negative and ER+ samples (r = 0.646 [95% CI 0.553–0.720]). There was 100% concordance for ER+ tumors; however, 56% of the ER-low+ tumors were negative by RT-PCR. Allred score correlated better with ER status measured by RT-PCR at pre-treatment (r = 0.83) than at post-treatment (r = 0.76). The majority (77%) of ER-negative and ER-low+ tumors were HER2-negative.

Conclusions: RT-PCR provided a more accurate assessment of ER expression in patients with ER-low+ tumors and data support dual testing for patients with ER-low+ status to ensure appropriate treatment planning as it pertains to endocrine therapy.

Keywords: 21-gene assay; Breast cancer; Endocrine therapy; Estrogen receptor; Gene expression; Immunohistochemistry; Pooled analysis; Proliferation; Recurrence Score; Reverse transcriptase-polymerase chain reaction.

INTRODUCTION

The estrogen receptor (ER) is a biomarker used to identify breast cancer patients who are most likely to benefit from endocrine therapy, such as tamoxifen or aromatase inhibitors (AIs) [1]. Current clinical practice guidelines, such as the National Comprehensive Cancer Network and the American Society of Clinical Oncology (ASCO), recommend that patients with ER-positive (ER+) early stage breast cancer receive at least 5 years of endocrine therapy and potentially up to 10 years of therapy in appropriate patients [2, 3]. Although time to disease recurrence is inversely related to ER expression level [4], even a very low level of ER expression is associated with a clinical benefit from endocrine therapy [5]. Furthermore, quantitative ER expression level has also been shown to predict distant recurrence and survival in patients treated with endocrine therapy [6].

The changing threshold for determining ER+ early stage breast cancer by immunohistochemistry (IHC) has presented a challenge to physicians and patients with respect to whether or not patients with lower levels of ER expression should be treated with endocrine therapy. Most recently, the ASCO/College of American Pathologists (CAP) guidelines have lowered the threshold from > 10% to > 1% by IHC staining [5]. Current clinical practice utilizes this ER threshold (> 1% staining) or Allred score ≥ 3 for determination of endocrine therapy eligibility [5, 7]. As a result, approximately 70% of patients are now being classified as ER+ and appropriate for endocrine therapy [8]. While these guidelines have improved ER status assessment through the standardization of certain pre-analytic variables (i.e. fixation duration; antibody choice), a 1% threshold may be too difficult to accurately replicate using IHC techniques to classify patients as ER+ or ER-negative. Recent studies have found that the majority of patients with < 10% ER

expression by IHC, similar to patients with ER-negative breast cancer, do not benefit from endocrine therapy [9–11]. Further, approximately 15–20% of patients have amplification (positive) of the human epidermal growth factor receptor 2 (HER2) [12], and are less likely to be responsive to endocrine therapy and require additional HER2 targeted therapy with monoclonal antibodies or kinase inhibitors for effective treatment [2].

Prognosis and treatment decisions are based on the level of ER expression, thus underscoring the need for an accurate quantitative assessment of ER, particularly in patients with tumors that have low levels of expression – in the 1–10% range (ER-low+). In general, women with ER+ tumors tend to have higher 5-year survival rates (85%) compared with women with ER-negative tumors (69%) [13]. Additionally, 5–10-year survival rates for women with ER+ early stage breast cancer are higher compared to ER-negative tumors across age groups [14]. ER-negative tumors tend to have a worse prognosis compared to ER+ tumors, are not responsive to endocrine therapies, and require a more aggressive approach usually with upfront chemotherapy [15]. It is estimated that approximately 75% of ER-low+ tumors are genetically similar to ER-negative tumors, and therefore unlikely to respond to endocrine therapy [16]. Thus, having greater precision of ER expression is critical to making the appropriate treatment plan for patients with low ER-expressing tumors by IHC.

Reverse-transcription polymerase chain reaction (RT-PCR) may provide a more precise indication of ER status. Classification of ER status by RT-PCR has been shown to have a >90% concordance with IHC [17–19]. Furthermore, in the National Surgical Adjuvant Breast and Bowel Project B-14 trial of patients with ER+ early stage breast cancer, ER measured by

quantitative RT-PCR was shown to be predictive of benefit from treatment with tamoxifen (interaction $p < 0.001$) [20]. To date, this is the only study that has shown a correlation between level of ER expression and response to endocrine therapy.

The 21-gene assay (Oncotype DX Breast Recurrence Score® assay, Genomic Health Inc., Redwood City, CA) is a multigene assay that employs quantitative RT-PCR-based methodology to generate a Recurrence Score result from RNA extracted from patient tumor tissue samples. The assay measures the expression levels of 21 genes, which is then used to calculate the Recurrence Score, measured on a continuous scale from 1 to 100 [21]. The 21-gene assay has been clinically validated to predict the 10-year risk of distant recurrence and the likelihood of benefit from chemotherapy in patients with ER+ early stage breast cancer who are treated with endocrine therapy [21–24]. The 21-gene assay also reports quantitative single gene scores for ER and progesterone receptor (PR), providing further insight into a patient's breast tumor biology and thus their likelihood of response to endocrine therapy.

IHC methods may be too sensitive at lower expression levels and result in misclassification of some patients as having ER+ early stage breast cancer. The aim of the current study was to compare ER expression measured by IHC and by RT-PCR (via the 21-gene assay) and compare the association between ER levels and endocrine therapy effect in patients with early stage breast cancer and ER-negative (< 1%) or ER-low+ (1–10%) expression by IHC.

METHODS

Two independent, but similarly designed, studies examined ER status determined by IHC and RT-PCR: Study 1 was a prospectively designed exploratory study of archived formalin fixed paraffin embedded tumor specimens from a consecutively accrued cohort of 140 patients with ER-negative (IHC < 1%) or low+ (IHC 1–10%) early stage breast cancer from New York University **(see Text S1 and Table S1a in the electronic supplementary material)** [25]. ER protein was assessed by standard IHC methods using the CONFIRM anti-ER (SP1) antibody (Ventana Medical Systems®, Tucson, AZ). HER2 protein assessment used standard IHC methods with the anti-HER2/neu (4B5) antibody (Ventana Medical Systems®, Tucson, AZ) with reflex testing of HER2 2+ cases by fluorescence in situ hybridization (FISH) (positive defined as ratio ≥ 2.0) using the FDA-approved HER2 DNA Probe Kit, Vysis FISH chromosome search (Abbott Molecular, Abbott Park, Illinois). FISH Hybridization results were recorded and analyzed by BioView Duet system (Allegro Plus Automated Scanner) with HER2 application software (BV-HER2-AF). Quantitative HER2 levels were obtained from the RT-PCR assay (via the 21-gene assay) as previously described [21, 26]. Study 2 included 55 postmenopausal women with low ER-expressing early breast cancer (Allred scores 2–7), who were treated as part of a prospectively randomized trial with 2 weeks of endocrine therapy with letrozole or anastozole, followed by wide excision, at the Edinburgh Breast Unit **(see Text S2 and Table S1b in the electronic supplementary material**) [27]. Since the data across these two studies included ER quantification by IHC and RT-PCR in primary tumor tissue samples of patients with early stage breast cancer, we pooled the two datasets to increase the power of the analysis. All ER (by IHC and RT-PCR) and HER2 (by RT-PCR) results were assessed per ASCO/CAP guidelines that control for pre-analytic, analytic, and post-analytic variables [5, 28].

Pooled Analysis: ER Quantification (IHC vs. RT-PCR)

Tumor tissue from patients with early stage breast cancer where ER status could be determined by both IHC assay and RT-PCR were included in the analyses. Samples were excluded if the tumor tissue was not a primary tumor or if insufficient tissue was available for RT-PCR or IHC assay analysis. Specimens from patients with early stage breast cancer who were identified as ER-negative or ER-low+ by IHC were included in the analysis. ER status by IHC staining was defined as: < 1% (ER-negative), 1–10% (ER-low+), or > 10% (ER+). ER Status by RT-PCR was defined as ≤ 6.5 (ER-negative) or > 6.5 (ER+). The 21-gene assay was run in the Clinical Laboratory Improvement Amendments certified/CAP accredited laboratory at Genomic Health, as per standard procedures. Recurrence Score results were reported on a continuous scale from 0 to 100, with risk classified as low (< 18), intermediate (18–30), or high (≥ 31) [21].

Association Between ER Level and Prediction of Endocrine Therapy Effect in Patients with ER-low+ Status (by IHC)

A subgroup analysis in postmenopausal women with Allred score 2–7 from Study 2 examined the association between ER expression and response to a 14-day pulse pre-surgery with an AI (letrozole or anastrozole), followed by wide excision. Pre-treatment Allred score (as determined by IHC analysis) and pre- and post-treatment 21-gene assay results were obtained for each sample. For all analyses, specimens were grouped into Allred score 2–4 (ER-low+) or Allred score 5–7 (ER+). Proliferation was measured by either Ki67 expression, a protein strongly associated with cell proliferation [29], using IHC methods or the 21-gene assay based proliferation gene group score (PGS). Proliferation response was defined as a ≥ 20% relative decrease in Ki67 expression, which was compared against any decrease in PGS [27].

Statistical Considerations

ER expression by RT-PCR and IHC was described and the concordance between ER classification by IHC and RT-PCR was calculated. Spearman's rank correlation coefficient and 95% confidence intervals [CIs] were calculated for ER expression by RT-PCR and ER percent by IHC, and for Recurrence Score result and ER expression by both RT-PCR and ER percent by IHC. Distribution of ER by both RT-PCR and by IHC were described among low, intermediate, and high Recurrence Score risk groups.

Study 2 analyzed changes in ER status and Ki67 expression, which were assessed using a paired t-test; statistical significance was set as $p < 0.05$ [27]. Correlations and 95% CIs for pre- and post-treatment measurements were calculated using Spearman rank statistics. Univariable logistic regression was used to test predictors for significant changes in Ki67 by IHC using the following co-variates: age; tumor size; Allred score (5–7 vs. 2–4); Recurrence Score; ER expression by RT-PCR; ER status by RT-PCR (positive vs. negative); PR expression by RT-PCR; PGS; Ki67 expression by RT-PCR; and HER2 expression by RT-PCR. Generalized linear models were used to test predictors for change in the proliferation axis score. All analyses were conducted using SAS v9.3 (SAS Institute, Cary, NC).

Association of HER2 and ER Expression in Patients with ER-Negative or ER-low+

A sub-analysis from Study 1 examined the association between low/negative ER expression and HER2 expression. HER2 status was established via ASCO/CAP guidelines [30]. Reference-normalized expression measurements ranged from 2 to 15, where each 1-unit increase reflects

about a two-fold increase in RNA and HER2 expression. Categories were based on pre-specified reference-normalized values (positive ≥ 11.5, equivocal ≥ 10.7 to < 11.5, negative < 10.7).
All analyses were descriptive in nature and included the distribution of HER2 expression by RT-PCR and the distribution of the Recurrence Score result across HER2 status by IHC/FISH. HER2 categorization by IHC/FISH and RT-PCR were compared and positive agreement between methods were calculated, which included equivocal results.

This article does not contain any new studies with human or animal subjects performed by any of the authors.

RESULTS

Pooled Analysis: Comparison of ER Status by IHC and RT-PCR

Overall, tumor specimens from 192 patients (median age 59 [range 25–92] years) were included in the pooled analysis **(Table 1).** The majority of tumors were grade 3 and had high (≥ 31) Recurrence Score results (86%).

Overall, the majority of samples (69%; 133/192) were ER-negative (< 1%) by IHC with 20% (39/192) classified as ER-low+ (1–10%) and 10% (20/192) as ER+ (> 10%) **(see Table 1)**. In Study 1, 76% (106/140) of samples were ER-negative, 24% (34/140) were ER-low+, and 0% (0/140) were ER+. In Study 2, 52% (27/52) of samples were ER-negative, 10% (5/52) were ER-low+ (Allred score 2–4), and 38% (20/52) were ER+ (Allred score 5–7). There was a distribution of ER expression measured by RT-PCR, within the IHC defined groups of ER-negative, ER-low+, or ER+ (**Fig. 1**). The correlation between IHC- and RT-PCR-measured ER **(Fig. 2a–d)**

was strong for IHC defined ER-negative and ER+ samples (r = 0.646 [95% CI 0.553–0.720]) (**Fig. 2a**). There was a 92% concordance between RT-PCR and IHC in classifying ER-negative status samples **(Fig. 2b)**, but only a 63% concordance in classifying samples as ER+. When stratified by ER-low+ (1–10% IHC staining; **Fig. 2c**) or strong ER+ (> 10% IHC staining; **Fig. 2d**), the concordance was 100% for tumors that were ER > 10%, but only 44% (17/39) of ER-low+ tumors were positive by RT-PCR. Conversely, more than half (56%) of the ER-low+ (1–10%) tumors were negative by RT-PCR (≤ 6.5).

There was a range of Recurrence Score values across levels of ER expression by IHC **(Fig. 3)** and RT-PCR **(see Fig. S1 in the electronic supplementary material)** with the majority of samples having high Recurrence Score results. Virtually all of the ER-negative samples by both IHC and RT-PCR had high Recurrence Score results (**Fig. 1**). None of the samples that were ER-negative by either method, or ER-low+ had a low Recurrence Score result (**Fig. 2b–c** and **Fig. 3 and 4)**. Of the patient samples with high Recurrence Score results, 79% (by IHC) and 86% (by RT-PCT) were ER-negative, 19% (by IHC) were ER-low+, and 2% (by IHC) and 14% (by RT-PCR) were ER+. There were 3/133 (2%) intermediate scores in the IHC ER-low+ tumors and 2/144 (2%) intermediate scores in tumors negative by RT-PCR (≤ 6.5). The Recurrence Score result correlated more closely to ER expression by RT-PCR compared with ER expression by IHC (r = -0.54; 95% CI -0.63 to -0.43 and r = -0.38; 95% CI -0.50 to -0.25, respectively) **(see Fig. S2a–b in the electronic supplementary material)**. ER expression tended to be higher in patients with a low Recurrence Score result and lower in patients with a high Recurrence Score result, regardless of method (**Fig. 4a–b**). However, within each Recurrence Score result-defined risk group (low, intermediate, high) there was a range of ER expression values.

Response to 14-day Pulse of an AI: ER Expression, Recurrence Score Result, Proliferation, and Ki67

A sub-analysis examined the effect on ER expression (by RT-PCR), Ki67 expression (relative and absolute), and the PGS from the 21-gene assay in 55 patients with Allred score 2–7 who had received 14 days of preoperative AI treatment. At pre-treatment, 45% of patients were Allred score 2–4 and 55% were Allred score 5–7. ER expression by RT-PCR correlated strongly with Allred score (r = 0.83; 95% CI 0.73–0.90) at pre-treatment, and declined, though remained strongly correlated, at post-treatment (r = 0.76; 95% CI 0.61–0.85). Pre- and post-treatment ER expression by RT-PCR were highly correlated (r = 0.88; 95% CI 0.79–0.93) and there was no statistically significant change in the mean (standard deviation [SD]) ER expression following AI therapy (6.8 [2.5] vs. 6.7 [2.3], respectively, $p = 0.47$ **[see Fig. S3 in the electronic supplementary material]**). Similarly, pre- and post-treatment Recurrence Score results were also highly correlated (r = 0.93; 95% CI 0.88–0.96), with eight patients (15%) changing risk group, three of whom decreased in risk group (two from intermediate to low; one from high to intermediate), following AI treatment **(see Fig. S4 in the electronic supplementary material**).

Pre-treatment Ki67 values were available for 45 (81.8%) samples. Spearman correlations between Ki67 by IHC and PGS were moderate in both pre-treatment samples (r = 0.37; 95% CI 0.08–0.59) and post-treatment samples (r = 0.54; 95% CI 0.28–0.71) **(see Fig. S5a–b in the electronic supplementary material).** There was a significant decrease in mean Ki67 level from pre-treatment (18%) to post-treatment (11%; $p < 0.001$) and the 64% of tumors with no change in Ki67 level all had high Recurrence Score results. Pre- and post-treatment PGSs were highly

correlated (r = 0.72; 95% CI 0.56–0.83) and there were clear differences between tumors with Allred score 2–4 versus Allred score 5–7 **(see Fig. S6 in the electronic supplementary material)**. Samples with an Allred score 2–4 showed minimal effect of the AI pulse with a mean (SD) change in PGS of 0.07 (0.29), while samples with an Allred score 5–7 showed a mean (SD) pre- to post-treatment change in PGS of -0.67 (0.71) **(see Fig. S7 in the electronic supplementary material).**

Of the 45 samples with a pre-treatment Ki67 value available, 28 (62%) had a decrease in both Ki67 and PGS after AI treatment. Compared with changes in Ki67 expression, PGS changes were more strongly correlated with Recurrence Score result **(Fig. 5a-b)**. Samples in the Allred score 5–7 group had the greatest absolute change in PGS **(see Fig S7 in the electronic supplementary material)**. Most (88.9%) tumors had an absolute decrease in Ki67 by IHC following AI treatment **(see Fig S8a in the electronic supplementary material)**. Pretreatment PGS did not predict the change in Ki67 on an absolute or relative scale **(see Fig S8b in the electronic supplementary material)**. There was greater relative reduction in both Ki67 expression and PGS in Allred score 5–7 (73% and 11%, respectively) than Allred score 2–4 (28% and -1%, respectively) tumors **(see Fig. S9 in the supplementary material)**. By univariate logistic regression analysis, Allred score 5–7, Recurrence Score result, ER by RT-PCR, ER+ status by RT-PCR, and PR by RT-PCR were all statistically significant predictors of change in Ki67 expression ($p < 0.01$ for each) and PGS ($p < 0.0001$ for each) (**Table 2**). Age, tumor size, PGS, Ki67 by RT-PCR, and HER2 by RT-PCR were not significant indicators of proliferation change ($p > 0.05$ for each).

HER2 Association with ER Expression in Patients with ER-Negative or ER-low+ Status (by IHC)

A third sub-analysis examined the association between HER2 expression in patients with ER-low+ or negative tumors. HER2 status was determined from tissue samples from 140 patients who were ER-negative (76%) or low ER+ (24%) from Study 1 **(see Table S2 in the electronic supplementary material)**. The percent positive agreement between HER2 as measured by RT-PCR and IHC/FISH was 78% when equivocal cases by RT-PCR were included in the calculation (82% when equivocal values were excluded). There was a high level of agreement between HER2 IHC methods and RT-PCR for ER-negative samples. The majority (75%) of ER-negative and ER-low+ tumors were HER2-negative by RT-PCR **(see Table S3 in the electronic supplementary material**).

DISCUSSION

In this report, we have examined the correlation between IHC and RT-PCR methods of ER assessment in a predominantly lower ER population as defined by using IHC for ER protein expression (< 1%, 1–10% and > 10%). We also explored the correlation between these low ER expressing carcinomas and the Recurrence Score result, described the effect of a 14-day neoadjuvant pulse of an AI on ER expression assessed by both RT-PCR and IHC, and examined associations with Ki67 expression by IHC, the PGS by RT-PCR and the Recurrence Score result. ER expression by RT-PCR showed a high correlation with ER expression in samples that were either ER-negative or ER+ (≥ 10% staining) by IHC **(see Table 1)**. One-hundred percent of samples classified as ER+ (> 10%) by IHC methods were ER+ by RT-PCR. However, the clinically relevant finding was that in more than half (56%) of samples classified as ER-low+ by

IHC (1–10% staining) assessment by RT-PCR showed them to be ER-negative. These findings are consistent with those of Iwamato et al. [16], which showed that 76% of ER-low+ samples by IHC were negative by mRNA-based methods and had gene signatures consistent with ER-negative samples. This finding suggests that patients with low ER expression by IHC ER-low+ status may derive little or no benefit from the addition of endocrine therapy.

The threshold for defining ER+ status and eligibility for endocrine therapy has been shifting over the past decade. Previously an IHC staining level of ≥ 30% was the threshold, which then decreased to ≥ 10%, and most recently guidelines from ASCO and CAP now recommend that ER positivity should be considered above a 1% threshold [5]. Approximately 6% of tumors have low+ or borderline ER status (1–10% ER staining by IHC) [16, 31] and our results suggest that IHC may be less accurate in determining true ER expression levels in these low+ tumors with contemporary IHC techniques, which presents a greater challenge to physicians and patients when making decisions regarding appropriate treatment [16]. However, accurate assessment of ER expression is critically important to ensure that patients with early stage breast cancer are treated appropriately. ER is both an important predictive and prognostic biomarker. Higher ER expression is reflective of the likelihood of responding to endocrine therapy, and patients with lower quantitative ER expression treated with endocrine therapy have higher recurrence rates compared with patients with higher ER expression [4, 9, 13, 16]. Consequently, accurate identification of ER expression is paramount for determining which patients are likely to benefit from endocrine therapy and which patients have insufficient levels of ER expression to derive any benefit from endocrine therapy and will suffer from avoidable side effects, as well as needing a more aggressive approach to their treatment. Our results show that RT-PCR provides

a quantitative, objective and precise assessment of ER expression in patients with ER-low+ tumors.

The 21-gene assay has been incorporated into clinical practice for over a decade. The 21-gene assay assigns a Recurrence Score result along a continuum from 0–100 to aid clinicians in determining whether chemotherapy is necessary in patients with ER+ early stage breast cancer. The prospective phase 3 Assigning Individualized Options for Treatment (TAILORx) trial, demonstrated that women with ER+, node-negative breast cancer and low Recurrence Scores (0–10) (n = 1619), who were treated with endocrine therapy alone, had a 9-year freedom from recurrence rate of 95.0% and an overall survival rate of 93.7% [32]. Further, endocrine therapy alone was non-inferior to chemotherapy in 6711 patients who had an intermediate Recurrence Score (11–25). In our study ER expression was highly correlated with the Recurrence Score results. Response to a 2-week pulse of an AI was determined by changes in Recurrence Score result, and proliferation (measured by the PGS by RT-PCR or Ki67 by IHC). The Recurrence Score result was a strong indicator of which patients had a proliferation response, with a decrease in PGS in low and intermediate scores, but not in patients with a high Recurrence Score result. Ki67 protein level decreased after the AI pulse, but there was greater variability in the relative decrease within the low and intermediate Recurrence Score groups, likely due to the greater variability and difficulty in quantitating Ki67 by IHC [33, 34]. Changes in PGS (measured using the 21-gene assay) were also seen after a pulse of AIs, showing that changes in PGS could be used to assess short-term endocrine sensitivity. Our AI Pulse substudy also showed that among the 55 patients included, the vast majority (92%) of ER-low+ (Allred score 2-4) tissue samples were ER-negative by RT-PCR. In contrast, 90% of samples with Allred score 5–7 were ER+.

Allred score correlated better with ER expression measured by RT-PCR at pre-treatment (r = 0.83) than at post-treatment (r = 0.76). Finally, 23% of patients with ER-low+ or ER-negative tumors by IHC were HER2+ by RT-PCR showing a high level of agreement in between RT-PCR and IHC/FISH measurements.

The strengths of this study include participation by several leading academic centers for high quality performance and interpretation of the IHC and for using a high-quality central laboratory with standardized methods for RT-PCR and performance of the 21-gene assay consistent with the TAILORx experience. Additionally, the results support other reports that RT-PCR can separate patients into ER+ and ER-negative status in ER-low+ tumors (1–10% by IHC) [9]. The analysis of the two datasets suggests that dual testing with IHC and RT-PCR for breast cancers with low ER expression may be useful for accurate ER assessment to facilitate therapeutic decisions. A similar algorithm is routinely utilized for HER2 assessment for cases with equivocal results (IHC/FISH). Finally, this study, showing decreases in Ki-67 IHC expression following a brief course of neoadjuvant endocrine therapy without chemotherapy, is supportive of previous data showing that the Recurrence Score result is associated with clinical response to neoadjuvant letrozole in postmenopausal patients with ER+, HER2-negative, clinically node-negative breast cancer [20, 35–37]

Limitations include that the samples were not from randomized clinical trials, the analyses were descriptive and correlative and there were not any clinical outcomes, although they do reinforce earlier reports that ER measured by RT-PCR and IHC are concordant in clearly ER+ (> 10%)- and ER-negative tumors [17, 38].

In summary, the value of the Recurrence Score assay result includes identifying many low risk patients who will not benefit from the addition of chemotherapy and precise and reproducible estimates of quantitative ER gene expression, in addition to the RS, may help guide neoadjuvant endocrine treatment decision making. These data support that single gene ER expression from the 21-gene assay can more precisely stratify IHC ER-low+ (1–10%) tumors into ER positive and negative groups in order to aid physicians and patients in optimal treatment planning.

CONCLUSION

Accurate measurement of ER is essential for optimal treatment of patients with early stage breast cancer. In patiets with ER-low+ tumors by IHC, additional testing may be valuable to assess ER status more accurately. Based on the results of this pooled analysis from two independent studies, a 1% threshold by IHC to determine ER+ status, as recommend by current treatment guidelines, appears to be too low. The current study and a review of previous reports suggest a threshold for determining ER+ status by IHC methods should be 10% [9, 11]. The current pooled analysis supports the value of measuring ER by RT-PCR in patients with ER-low+ status to ensure optimal treatment planning of endocrine therapy, particularly in patients that are HER2-negative. In patients with lower ER by IHC, the 21-gene assay provides not only more precise information than IHC in patients with ER-low+ expression (1–10%) and can be used to determine which patients are likely to benefit from endocrine therapy, but also predicts their outcome and the need for alternative treatments such as chemotherapy.

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Prior presentations

Information in this article has been presented previously, in part, as posters:

- Sing A, Dixon JM, Turnbull A, et al. Association of estrogen receptor (ER) levels and prediction of antiproliferative effect of hormone therapy in lower ER-expressing tumors. 37th Annual San Antonio Breast Cancer Symposium (SABCS 2014); December 9-13, 2013; San Antonio, Texas. Abstract #P3-06-35.
- Singh B, Ziguridis N, Axelrod D, et al. Discordance in hormone receptor (HR) assessment by IHC and RT-PCR in an estrogen receptor (ER) low-positive group (1-10% positive cells): Does accurate assessment of HR status require dual testing? 36th Annual San Antonio Breast Cancer Symposium (SABCS 2013); December 10-14, 2013; San Antonio, Texas. Abstract #P3-05-08.
- Singh B, Ziguridis N, Butler S, et al. Comparison of HER2 testing by IHC/FISH and RT-PCR in estrogen receptor negative or borderline patients with early stage breast cancer. 102nd United States and Canadian Academy of Pathology (USCAP) Annual Meeting; March 2-8, 2013; Baltimore, Maryland. Abstract #282.

Authorship

All named authors meet the International Committee of Medical Journal Editors (ICMJE) criteria for authorship for this manuscript, take responsibility for the integrity of the work as a whole, and have given final approval to the version to be published.

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Disclosures

J. Michael Dixon, Laura M. Arthur, Deborah M. Axelrod, Lorna Renshaw, Jeremy S. Thomas, Arran Turnbull, and Oliver Young declare that they have no conflict of interest. David A. Cameron attended an advisory board for Genomic Health, Inc. (any income associated with the advisory board was paid to Dr. Cameron's employer and none to Dr. Cameron). Cynthia A. Loman is an employee of and declares stock ownership in Genomic Health, Inc. Debbie Jakubowski is an employee of and declares stock ownership in Genomic Health, Inc. Frederick L. Baehner is an employee of and declares stock ownership in Genomic Health, Inc. Baljit Singh is a member of the speakers' bureau for Genomic Health, Inc. The study sponsor, Genomic Health, Inc., funded the processing charges for this article.

Compliance with ethics guidelines.

This article is based on previously conducted studies and does not contain any studies with human or animal subjects performed by any of the authors. In Study 1 (New York University),

the link between patient identifiers and clinico/pathological data was permanently deleted and the study was 'exempt' from review by the New York University Internal Review Board. Patients in Study 2 (Edinburgh Breast Unit) were recruited under ethical approval from the South East Scotland Research Ethics Committee 03, approved in 2002, reference LREC/2002/8/23.

Data availability

All data generated or analyzed during this study are included in this published article/as supplementary information files.

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Figure Legends

Fig. 1 Estrogen receptor status by IHC[a] and RT-PCR in 192 patients with early stage breast cancer

[a]ER-low-positive = Allred score 2–4; ER+ = Allred score 5–7

ER estrogen receptor, *IHC* immunohistochemistry, *RT-PCR* reverse-transcriptase polymerase chain reaction

Fig. 2 Correlation of ER expression, measured by IHC and RT-PCR; overall (a), ER-negative (< 1% IHC staining) (b), ER low+ (1–10% IHC staining) (c), and ER+ (> 10% IHC staining) (d)

ER estrogen receptor, *IHC* immunohistochemistry, *RT-PCR* reverse transcriptase polymerase chain reaction

Fig. 3 Recurrence Score risk group, by IHC ER status (negative or positive) group

ER estrogen receptor, *IHC* immunohistochemistry, *RT-PCR* reverse-transcriptase polymerase chain reaction

Fig. 4 Distribution of ER expression by Recurrence Score risk group, by IHC and RT-PCR; IHC (a) and RT-PCR (b)

ER estrogen receptor, *IHC* immunohistochemistry, *RT-PCR* reverse-transcriptase polymerase chain reaction

Fig. 5 Changes in PGS and Ki67 expression, by pre- and post-treatment with AI, and Recurrence Score result; PGS (n=45)[a] (a) and Ki67 (n=45)[a] (b)

[a]The treatment effect is demonstrated by divergence from 0

AI aromatase inhibitor, *PGS* proliferation gene group score

Tables

Table 1 Baseline patient demographics and disease characteristics

Characteristic	***N* = 192**
Median age, years (range)	59 (25–92)
Mean tumor size, mm (SD)	19.3 (12.4)
Tumor grade, *n* (%)	
Grade 1	8 (4)
Grade 2	43 (22)
Grade 3	141 (73)
Tumor subtype, *n* (%)	
Ductal carcinoma, not otherwise specified	171 (89)
Lobular	9 (5)
Mixed/Other	11 (6)
Missing	1 (<1)
ER expression level by IHC, *n* (%)	
Negative (< 1% staining)	133 (69)
Low (1–10% staining)	39 (20)
Positive (> 10% staining)	20 (10)
Mean ER expression level, % (SD)	
IHC	7.94 (20.39)
RT-PCR	5.42 (1.98)
Recurrence Score risk group, *n* (%)	

Low	5 (3)
Intermediate	22 (11)
High	165 (86)
Median Recurrence Score (range)	54.74 (9.8–100)
HER2 status by IHC, *n* (%)	*N* = 140
0	36 (26)
1+	50 (36)
2+	21 (15)
3+	32 (23)
Missing	1
HER2 status by IHC and FISH, *n* (%)	*N* = 140
Negative	99 (71)
Positive	41 (29)
HER2 status by RT-PCR, *n* (%)	*N* = 140
Negative	105 (75)
Equivocal	3 (2)
Positive	32 (23)

ER estrogen receptor, *FISH* fluorescence in situ hybridization, *HER2* human epidermal growth factor receptor-2, *IHC* immunohistochemistry, *RT-PCR* reverse-transcriptase polymerase chain reaction, *SD* standard deviation

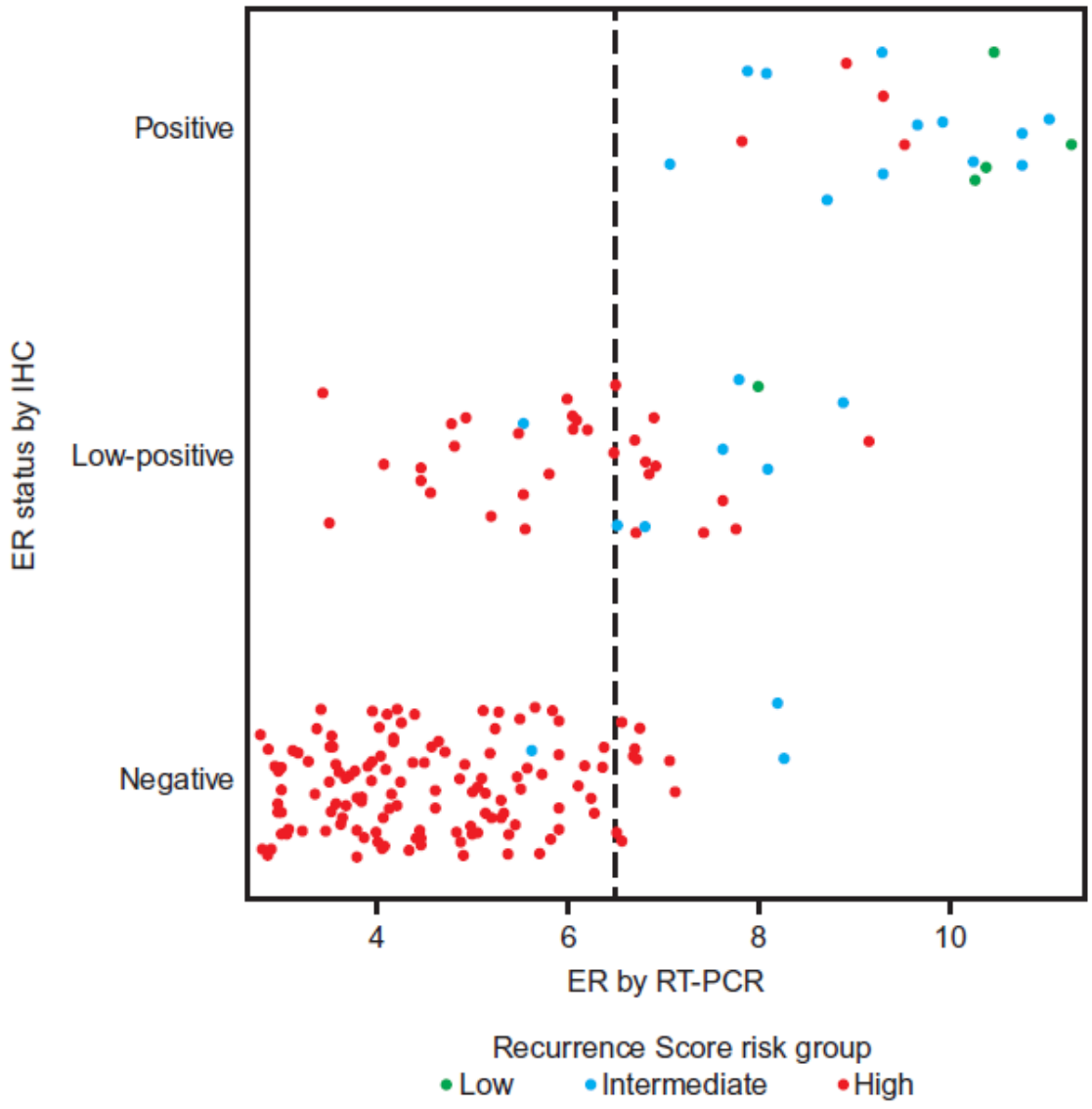
Table 2 Univariate logistic regression analysis evaluating predictors of proliferation change after 2 weeks of aromatase inhibitor therapy (Study 2)

Variable	**Proliferation measure (*p*-value)**	
	≥ 20% relative change in Ki67 expression[a]	**Decrease in PGS[b]**
Allred score 5–7 vs. 2–4	0.0017	< 0.0001
Recurrence Score result	0.0017	< 0.0001
ER expression, measured by RT-PCR	0.0019	< 0.0001
ER-positive, by RT-PCR	0.0029	< 0.0001
PR expression, measured by RT-PCR	0.0092	< 0.0001
HER2 by RT-PCR	0.0793	0.1297
Ki67 by RT-PCR	0.1689	0.979
Patient age	0.6165	0.1284
Tumor size	0.8589	0.7312

[a]Chi-Square Statistic, [b]T-test

ER estrogen receptor, *HER2* human epidermal growth factor receptor-2, HER2 *PGS* proliferation gene group score, *PR* progesterone receptor, *RT-PCR* reverse-transcriptase polymerase chain reaction

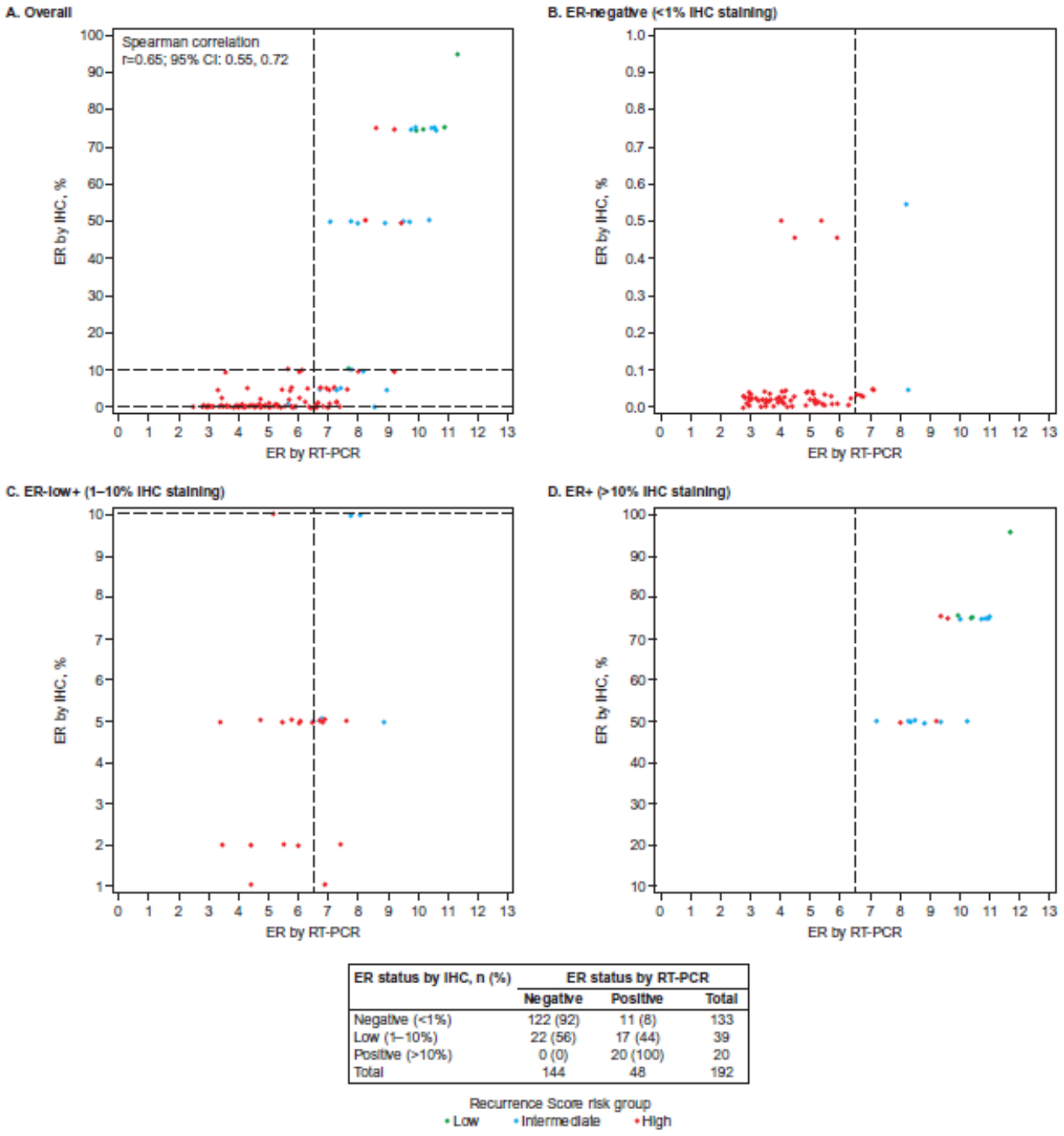
Fig. 1 Estrogen receptor status by IHC[a] and RT-PCR in 192 patients with early stage breast cancer



[a]ER-low-positive = Allred score 2–4; ER+ = Allred score 5–7

ER estrogen receptor, *IHC* immunohistochemistry, *RT-PCR* reverse-transcriptase polymerase chain reaction

Fig. 2 Correlation of ER expression, measured by IHC and RT-PCR; overall (a), ER-negative (< 1% IHC staining) (b), ER low+ (1–10% IHC staining) (c), and ER+ (> 10% IHC staining) (d)

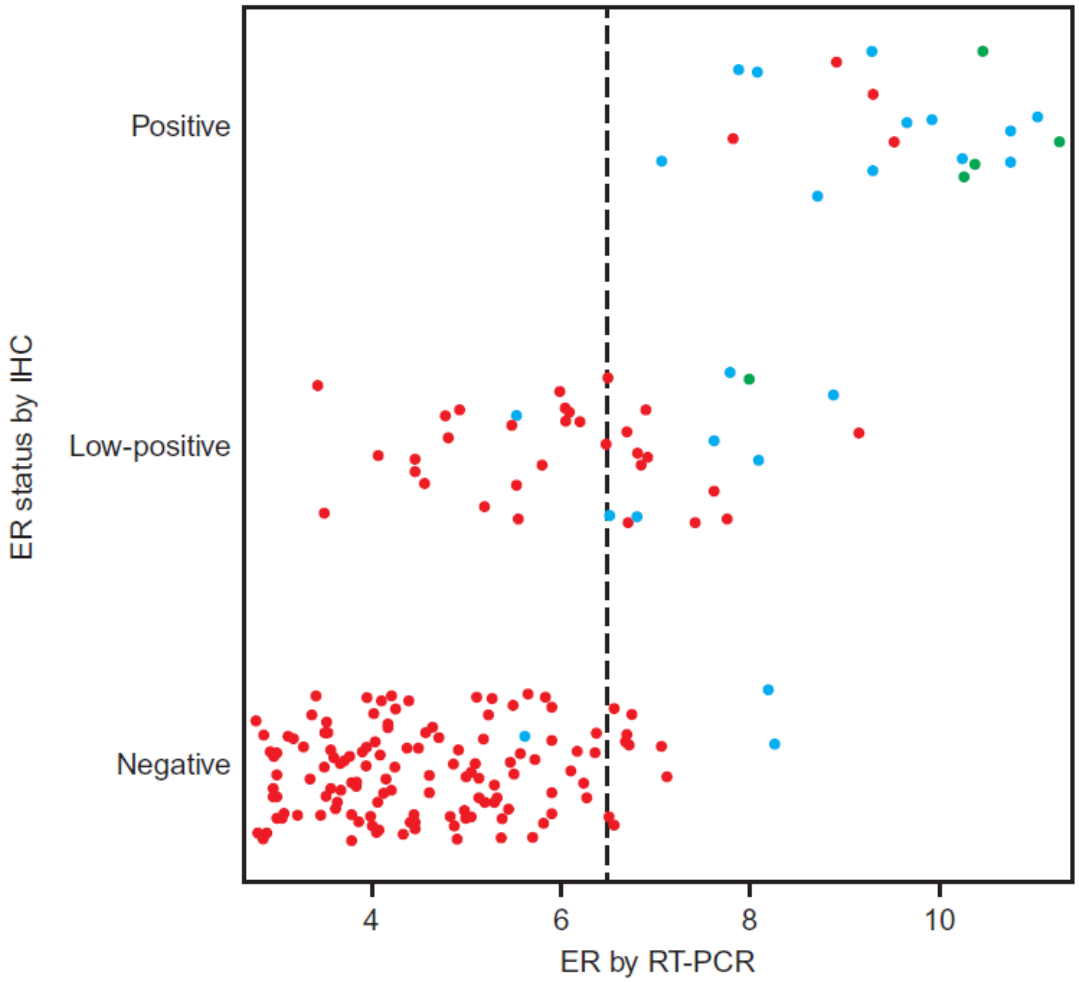


ER status by IHC, n (%)	ER status by RT-PCR		
	Negative	Positive	Total
Negative (<1%)	122 (92)	11 (8)	133
Low (1–10%)	22 (56)	17 (44)	39
Positive (>10%)	0 (0)	20 (100)	20
Total	144	48	192

Recurrence Score risk group
• Low • Intermediate • High

ER estrogen receptor, *IHC* immunohistochemistry, *RT-PCR* reverse transcriptase polymerase chain reaction

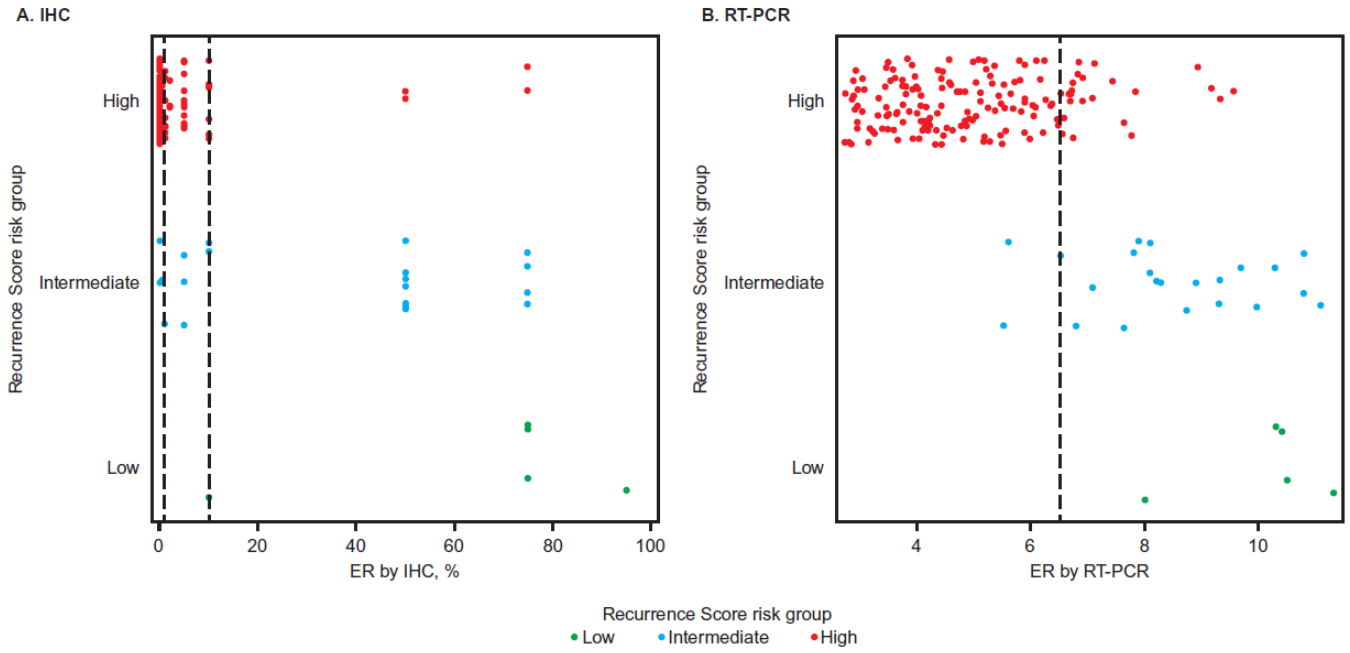
Fig. 3 Recurrence Score risk group, by IHC ER status (negative or positive) group



Recurrence Score risk group, n (%)	ER by IHC			Total
	Negative (<1%)	Low (1–10%)	Positive (>10%)	
Low	0 (0)	1 (20.0)	4 (80.0)	5
Intermediate	3 (13.6)	7 (31.8)	12 (54.6)	22
High	130 (78.8)	31 (18.8)	4 (2.4)	165
Total	133	39	20	192

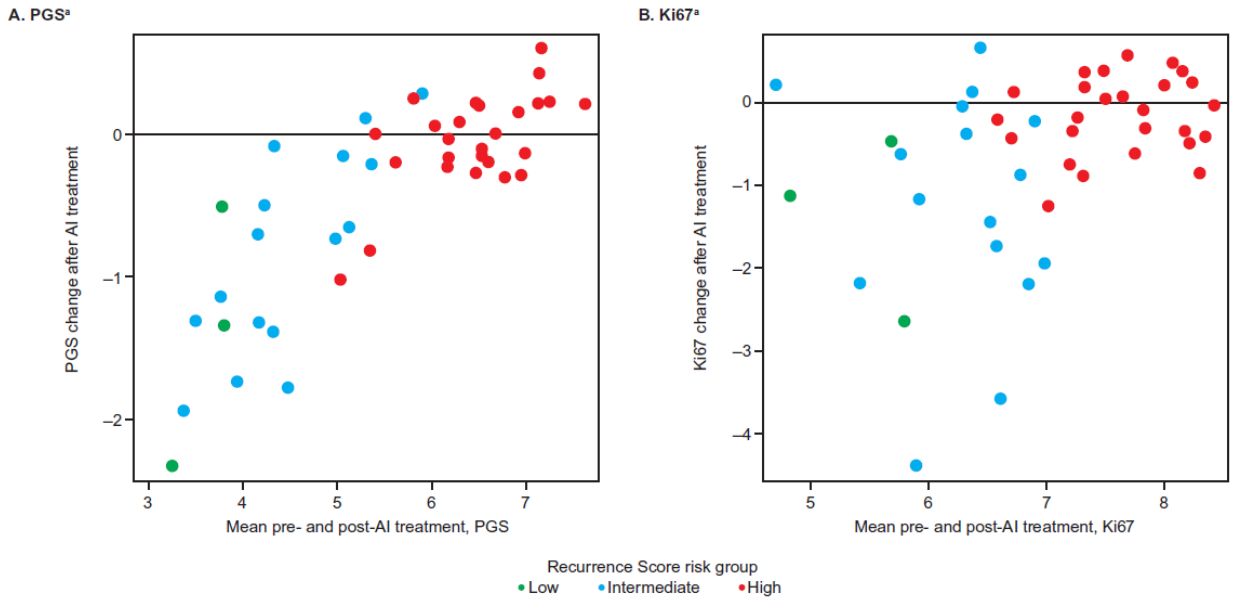
ER estrogen receptor, *IHC* immunohistochemistry, *RT-PCR* reverse-transcriptase polymerase chain reaction

Fig. 4 Distribution of ER expression by Recurrence Score risk group, by IHC and RT-PCR; IHC (a) and RT-PCR (b)



ER estrogen receptor, *IHC* immunohistochemistry, *RT-PCR* reverse-transcriptase polymerase chain reaction

Fig. 5 Changes in PGS and Ki67 expression, by pre- and post-treatment with AI, and Recurrence Score result; PGS (n=45)[a] (a) and Ki67 (n=45)[a] (b)



[a]The treatment effect is demonstrated by divergence from 0

AI aromatase inhibitor, *PGS* proliferation gene group score

SUPPLEMENTARY MATERIAL

Text S1 Protocol for Study 1 (New York University [NYU] study)

Overall Study Design

This was a prospectively designed exploratory study of archived formalin fixed paraffin embedded (FPET) tumor specimens from a consecutively accrued cohort of patients from NYU. Starting from the most current materials and proceeding in reverse chronologic order to identify eligible cases, approximately 140 case (approximately 100 estrogen receptor (ER)-negative and approximately 40 ER-borderline cases were to be identified. The study was observational with no impact on patient treatment or outcome.

Patient Risks and Benefits

There were no patient risks or benefits associated with this study protocol. All tissue specimens were de-identified prior to sending to Genomic Health (GHI) for further analysis. Assay results were for research purposes only and were not used for patient care or treatment decisions.

Study Objectives

1. To explore the correlation between ER assessments by central immunohistochemistry (IHC) and central reverse-transcription polymerase chain reaction (RT-PCR) in IHC ER-negative (< 1% positive tumor cells) FPET breast tumors
2. To explore the correlation between ER assessments by central IHC and central RT-PCR in IHC ER-borderline (1–10% positive tumor cells) FPET breast tumors

Inclusion Criteria

- FPET tumor tissue from patients diagnosed with early stage invasive breast cancer (IBC)
- Eligible for analysis after administrative review
- Consecutive FPET blocks containing IHC ER-negative (< 1% tumor cells positive) or IHC ER-borderline (1–10% tumor cells positive) early stage IBC
- FPET IBC tumor that was available in the NYU pathology archive starting from most current materials and reviewing in reverse chronologic order to identify eligible cases

Exclusion Criteria

- Not primary breast tumor
- Insufficient IBC (< 2.0 mm) in specimen as assessed by examination of the hematoxylin & eosin (H&E) slide by the GHI designated pathologist
- No IHC assessment of ER available
- Failure of pre-specified laboratory metrics

Specimen Handling, Blinding, and Testing

Once identified, specimens were sent to GHI for 21-gene Oncotype DX Breast Recurrence Score® assay (21-gene test; Genomic Health, Redwood City, CA) testing. GHI performed the 21-gene test while blinded to the NYU patient data. Both teams (NYU and GHI) conducted the analyses in parallel and jointly reviewed the results.

<u>Patient and Specimen Identifiers</u>

NYU ensured an accurate link between the unique patient identifier (PID) assigned by NYU and data collected by NYU for each patient included in the study. The PID for each patient specimen was recorded on the Specimen Handling Form that was sent to GHI. The PID was provided to GHI without any information that would allow identification of the individual such as name, initials, or medical record number.

Specimen Specifications

All FPET blocks submitted had the patient pathology reports reviewed in order to select the same sample used for IHC by the Pathologist at NYU to identify all blocks for the 21-gene assay.

The FPET blocks were provided such that each sample yielded a total of 13 x 5 μm unstained slides, from which the last slide was H&E stained to provide a guide slide for manual micro-dissection, should micro-dissection prove necessary. H&E for each patient was scanned and the digital image stored. The H&E was returned after completion of sample processing. In addition to being the same sample block used for IHC, patient tissue blocks provided to GHI were the most representative, e.g. to contain the largest cross sectional area of invasive breast carcinoma.

All residual RNA, beyond that needed for this study and for quality control or regulatory purposes, was retained by GHI. Remaining blocks were sent back to the pathologist.

Sample Preparation by GHI

- The GHI designated Pathologist microscopically reviewed the H&E to confirm the presence and sufficiency of the IBC tumor; then, the tumor area was highlighted using indelible ink on the glass H&E slide when manual micro-dissection was necessary
- When necessary, the tumor and non-tumor elements were manually micro-dissected. The enriched tumor was put into microcentrifuge tube(s) for processing
- Cases with non-tumor metabolically active tissues, such as proliferative fibrocystic change, biopsy cavities, skeletal muscle or skin, were manually micro-dissected
- All excess materials were retained and banked at GHI for regulatory or assay quality control and assurance purposes. All residual RNA, beyond that needed for this study and for quality control or regulatory purposes, was retained by GHI. Remaining blocks were sent back to the pathologist

Pathology Assessment

To ensure uniformity and consistency in the acquisition of pathology data relevant to the pre-specified analyses for this study, the pathology data collection was performed on all specimens by an academic surgical pathologist(s) with expertise in breast carcinoma. For this study, GHI central pathology assessment was performed.

GHI Assay

RT-PCR for the 21-gene test was performed according to standard procedures. All laboratory analyses were performed by designated laboratory personnel blinded to specimen information, without knowledge of treatment assignment or clinical outcomes. Expression levels of 21 genes used in the calculation of the Recurrence Score were measured as continuous values from the

quantitative RT-PCR assay. Gene expression values were normalized relative to the mean of the 5 reference genes according to pre-specified procedures. Reference-normalized expression measurements typically range from 2 to 15, where a 1-unit increase reflects an approximate 2-fold increase in RNA expression. The Recurrence Score, calculated from the reference-normalized expression measurements, was reported for each evaluable sample on a scale from 0 to 100.

Pathology Requirements

The specimen must have had all of the following characteristics (as assessed by the GHI pathologist) to be processed:

a. IBC must be present
b. The percentage of invasive carcinoma cells must be ≥ 5% or > 2.0 mm in greatest dimension
c. IHC results for ER

And one of the following:

a. The specimen must be ≥ 50% tumor

OR

b. The specimen must be < 50% tumor and is amenable to dissection

Pathology Data Variables

- Pathology variables acquired from NYU Pathology database:
    - Histologic grade using revised Nottingham criteria
    - Histologic subtype

    - Tumor size (mm)
- H&E stained tissue sections examined by a GHI pathologist or pathology designee to collect standard commercial metrics

Laboratory Data Variables

- ***Gene Expression:*** Reference-normalized expression levels of 16 cancer-related genes used in the calculation of the Recurrence Score were reported as values from the GHI assay. For each cancer-related gene, cycle threshold (C_T) measurements were obtained by RT-PCR, and then normalized relative to a set of 5 reference genes according to standard procedure
- ***Gene Panel:*** The 21 pre-specified gene expression panel consists of 16 cancer-related genes (BAG1, Bcl2, CCNB1, CD68, CEGP1, CTSL2, EstR1, GRB7, GSTM1, HER2, Ki-67, MYBL2, PR, STK15, STMY3, SURV), used in the calculation of the Recurrence Score, and 5 reference genes (B-actin, GAPDH, GUS, RPLPO, TFRC), used to normalize gene expression

The positive/negative cutpoints on the reference-normalized C_T scale for ER, progesterone receptor (PR) and HER2 are shown in the **table** below (hormone receptor [HR]+ is defined as ER+ and/or PR+; HR-negative is defined as both ER-negative and PR-negative):

Hormone receptor status genes and cutpoints (measured in reference-normalized C_T values)			
Gene	**HR**		
	Negative	**Equivocal**	**Positive**
ER	< 6.5	N/A	≥ 6.5
PR	< 5.5	N/A	≥ 5.5
HER2	< 10.7	10.7 to < 11.5	≥ 11.5

- ***Recurrence Score:*** The Recurrence Score on a scale from 0 to 100 was derived from the reference-normalized gene expression measurements. The Recurrence Score groups were defined as low (< 18), intermediate (18–30) and high (≥ 31)

Clinical Data Variables

Data collection was conducted under the direction of the primary investigator. In addition to the variables noted above, patient demographics and clinical data may have included the following:

1. ER status by IHC (intensity and percentage positive cells if applicable)
2. PR status by IHC (intensity and percentage positive cells if applicable)
3. HER2 status by IHC (intensity and percentage positive cells if applicable)
4. Patient age

Additional Data Handling

No GHI personnel had access to the un-blinded Clinical Data until after the GHI data were locked. Upon lock of GHI data, NYU transferred their data to GHI using GHI's secure web portal. Upon receipt of the NYU data, GHI merged the NYU data and the GHI data and both teams were able to conduct the analyses in parallel and jointly review obtained results.

Statistical Methods

GHI performed the 21-gene test while blinded to the NYU patient data. Both teams (NYU and GHI) were able to conduct the analyses in parallel and jointly review obtained results. Pre-specified assay metrics, as defined in the associated laboratory protocol, were used to identify the evaluable assay results. The distribution of ER single gene scores were characterized using descriptive statistics separately for the patients who were ER+ by IHC and those who were borderline ER+ by IHC. The proportion of evaluable patients who were ER+ by RT-PCR were calculated, along with the 95% confidence interval (CI) for the patients who were ER+ by IHC and those who were borderline ER+ by IHC.

Sample Size and Power

If 85% of 140 patients were ER+ by RT-PCR, the 95% CI for the proportion would be (70.2%, 94.3%). If 80% of 140 patients were ER+ by RT-PCR, the 95% CI for the proportion would be (64.4%, 90.9%).

Study Document Access and Availability, and Record Keeping

The primary investigator ensured the reliability and accuracy of the data provided to GHI, maintaining the confidentiality of the documents and tissue samples. The

Investigator(s)/Institution(s) permitted research-related monitoring audits, institutional review board (IRB)/EC review, and regulatory inspections by providing direct access to service data/documents. The investigator ensured the reliability and availability of the source. The primary investigator was responsible for maintaining adequate records to enable the conduct of the study to be fully documented.

Confidentiality and Ethical Issues

All tumor samples and reports were submitted to GHI without any information that would allow identification of the individual (i.e., name, social security number, and any number assigned by the hospital or medical office). Patient identification information were blocked out by the investigator prior to shipping to GHI. Reports and tissues were labeled with a serial number assigned by the primary investigator or designee, which enabled at the conclusion of the study linking assay data back to clinical data. The primary investigator was responsible for conducting this research protocol. The study was reviewed and approved by an Institutional Review Board/Ethics Committee and appropriate Scientific Review Committees prior to initiation, as required.

Text S2 Protocol for Study 2 (Edinburgh Breast Unit study)

Overall Study Design

This was a prospectively designed study in postmenopausal women with low estrogen receptor (ER)-expressing early breast cancers who were treated with an aromatase inhibitor (letrozole or anastrozole) for 2 weeks to determine if aromatase inhibitors reduced proliferation in low-ER-expressing cancers.

Study Aims

1. To correlate estrogen receptor (ER) levels by Allred score (immunohistochemistry [IHC]) vs. reverse transcriptase polymerase chain reaction (21-gene assay)
2. To describe changes in ER, the Recurrence Score result, and measures of proliferation after 2 weeks of treatment with an aromatase inhibitor
3. To perform exploratory analyses of factors associated with changes in proliferation

Patient Population

- Post-menopausal women with early breast cancer and lower ER (Allred score 2–7)

Treatment

- Two weeks of an aromatase inhibitor (letrozole or anastrozole) followed by wide excision

Methodology

- Only pre-treatment Allred scores were obtained

- All patients had a 21-gene assay on a pre-and post-treatment sample
- Proliferation was measured by Ki67 using IHC (in 45 patients) and by the proliferation gene group score (PGS) in the 21-gene assay (in all patients)
- Proliferation response was defined by a 20% relative decrease in Ki67 or any decrease in PGS
- Changes in proliferation (Ki67 and/or PGS) were correlated with Allred score, ER-PCR, and the 21-gene Recurrence Score result

Statistical Methods

- Correlations for measurements before and after treatment were calculated using Spearman rank statistics. For all analyses, patients were grouped by AS 2–4 or 5–7
- *p*-values for significant change in ER and Ki67 were obtained using a paired t-test
- Logistic regression was used to test predictors for significant change in Ki67
- Generalized linear models were used to test predictors for change in the proliferation axis score
- SAS version 9.3 statistical software was used

Ethics

Patients were recruited under ethical approval from the South East Scotland Research Ethics Committee 03, approved in 2002, reference LREC/2002/8/23.

SUPPLEMENTARY TABLES AND FIGURES

Table S1 Demographics and baseline characteristics of patients in Study 1 (A) and Study 2 (B)

A. Study 1 (New York University)

Characteristic	*n*	ER status, by IHC, *n* (%)	
	(*N* = 140)	Negative	Borderline
		(*n* = 106)	(*n* = 34)
Age, years			
< 50	43	32 (30.2)	11 (32.4)
50–59	36	28 (26.4)	8 (23.5)
60–69	30	25 (23.6)	5 (14.7)
≥ 70	31	21 (19.8)	10 (29.4)
Histologic grade			
Well differentiated	2	0 (0.0)	2 (5.9)
Moderately differentiated	21	11 (10.4)	10 (29.4)
Poorly differentiated	117	95 (89.6)	22 (64.7)
Positive nodes			
0	86	71 (75.5)	15 (48.4)
1–3	32	18 (19.1)	14 (45.2)
> 3	7	5 (5.3)	2 (6.5)
Tumor size, cm			
< 2	92	71 (67.0)	21 (61.8)
2 to < 4	41	28 (26.4)	13 (38.2)
> 4	7	7 (6.6)	0 (0.0)

PR status, by IHC			
Negative	116	96 (90.6)	20 (58.8)
Positive	24	10 (9.4)	14 (41.2)
HER2 status, by IHC			
0	36	28 (26.7)	8 (23.5)
1+	50	40 (38.1)	10 (29.4)
2+	21	14 (13.3)	7 (20.6)
3+	32	23 (21.9)	9 (26.5)
Missing	1	1	0
HER2-positive			
Yes	34	24 (22.6)	10 (29.4)
No (IHC 3+ or FISH > 2.2)	106	82 (77.3)	24 (70.5)

B. Study 2 (Edinburgh Breast Unit)

Characteristic	*n* (%) (*N* = 55)
Age, years	
< 50	1 (2)
50–59	17 (31)
60–69	19 (35)
≥ 70	18 (33)
Histologic grade	

Well differentiated	7 (13)
Moderately differentiated	20 (36)
Poorly differentiated	28 (51)
Tumor size, cm	
< 1	4 (7)
1–2	28 (51)
> 2	21 (38)
Missing	2 (4)
Tumor subtype	
Ductal	45 (82)
Lobular	5 (9%)
Other	5 (9%)
ER Allred score at baseline	
2	7 (13)
3	11 (20)
4	7 (13)
5	9 (16)
6	4 (7)
7	17 (31)

ER estrogen receptor, *FISH* fluorescence in situ hybridization, *HER2* human epidermal growth factor receptor-2, *IHC* immunohistochemistry, *PR* progesterone receptor

Table S2 HER2 status as measured by IHC/FISH and RT-PCR in patients with early stage breast cancer and ER-negative (*n* = 106, 76%) or ER-low+ (*n* = 34, 24%) expression (Study 1)

HER2 status by IHC FISH	HER2 status by RT-PCR, *n* (%)			
	Negative	Equivocal	Positive	Total
Negative	98 (99)	1 (1)	0	99 (100)
Positive	7 (17)	2 (5)	32 (78)	41 (100)
Total	105 (75)	3 (2)	32 (23)	140 (100)

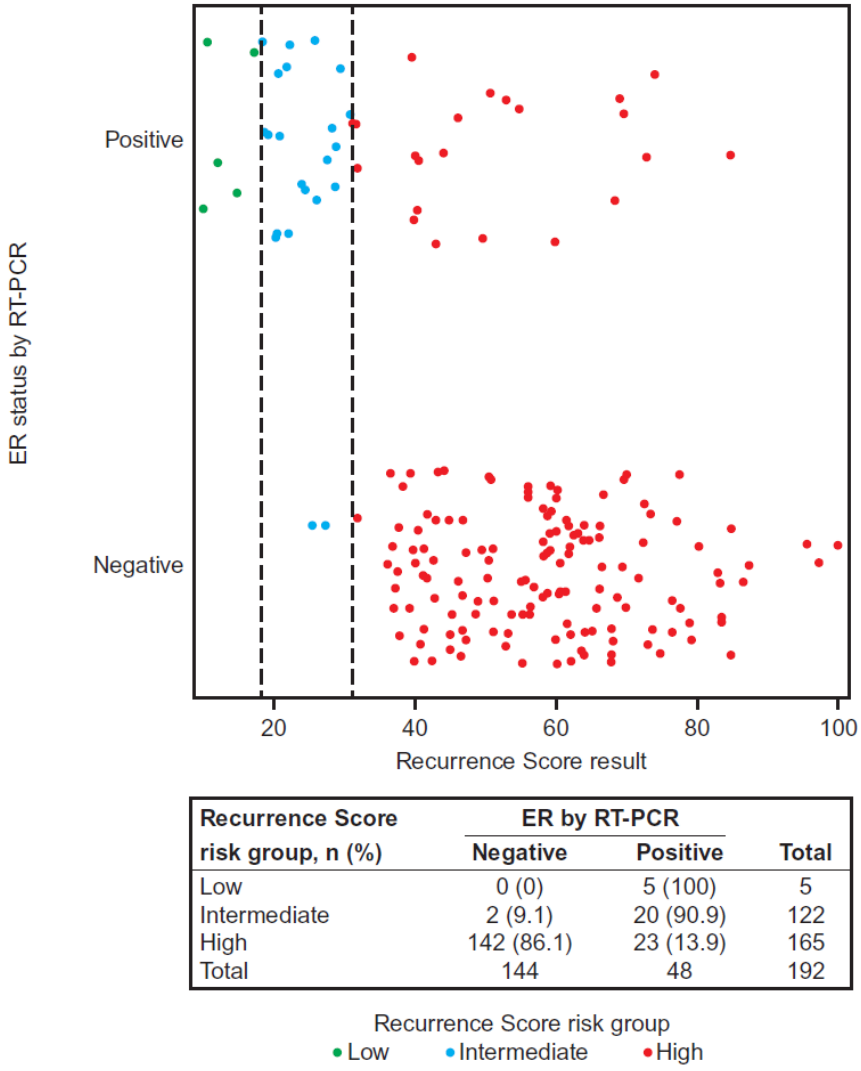
ER estrogen receptor, *FISH* fluorescence in situ hybridization, *HER2* human epidermal growth factor receptor-2, *IHC* immunohistochemistry, *NA* not applicable, *RT-PCR* reverse-transcriptase polymerase chain reaction

Table S3 HER2 status as measured by RT-PCR in patients with early stage breast cancer and negative or ER-low+ expression (Study 1)

ER status (by IHC)	HER2 status (by RT-PCR), *n* (%)			
	Negative	Equivocal	Positive	Total
Negative	82 (77)	2 (2)	22 (21)	106 (100)
Low	23 (68)	1 (3)	10 (29)	34 (100)
Total	105 (75)	3 (2)	32 (23)	140 (100)

ER estrogen receptor, *HER2* human epidermal growth factor receptor-2, *IHC* immunohistochemistry, *RT-PCR* reverse-transcriptase polymerase chain reaction

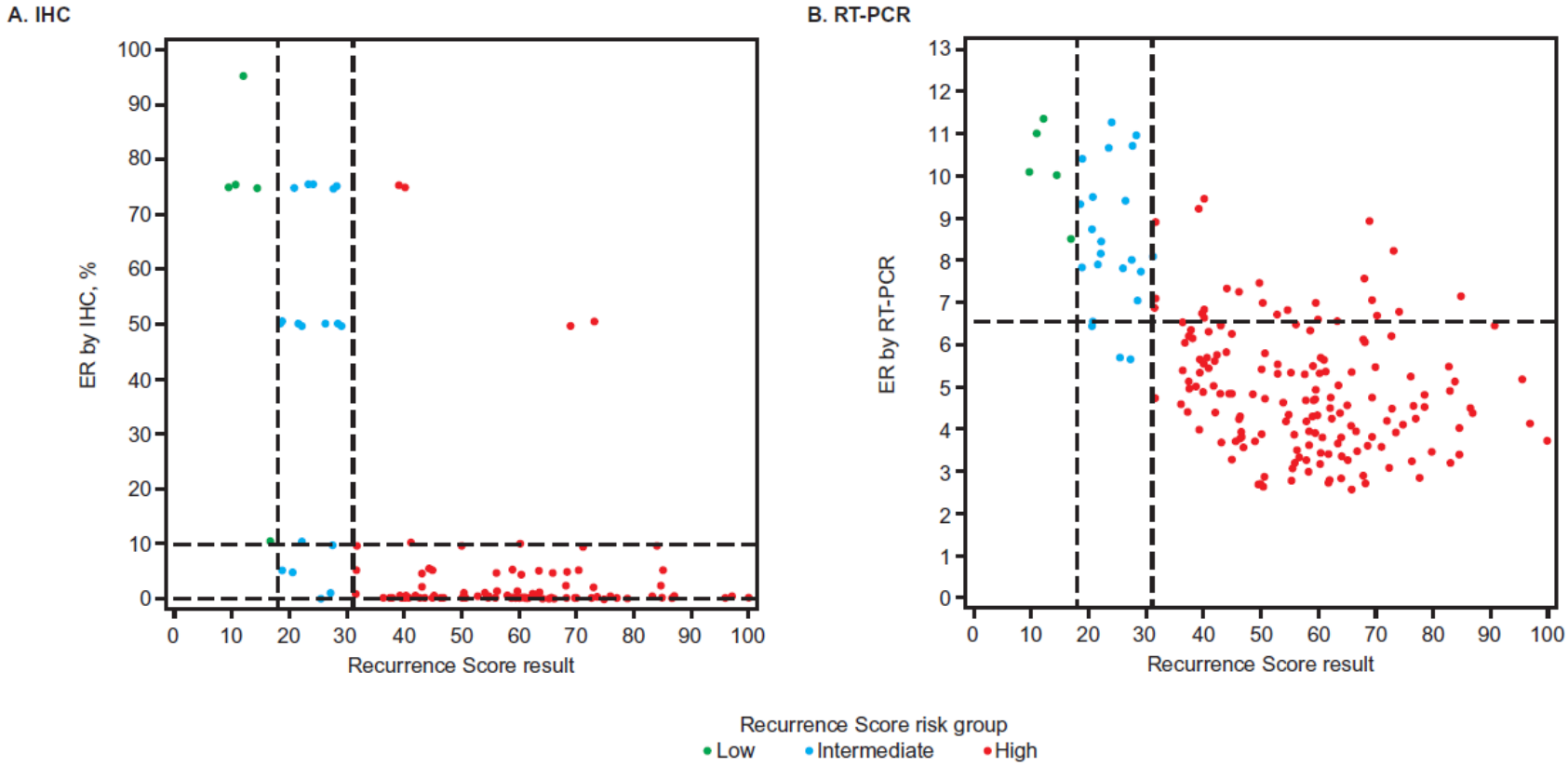
Fig. S1 Recurrence Score result group by RT- PCR ER expression group (negative or positive)



Recurrence Score risk group, n (%)	ER by RT-PCR		Total
	Negative	Positive	
Low	0 (0)	5 (100)	5
Intermediate	2 (9.1)	20 (90.9)	122
High	142 (86.1)	23 (13.9)	165
Total	144	48	192

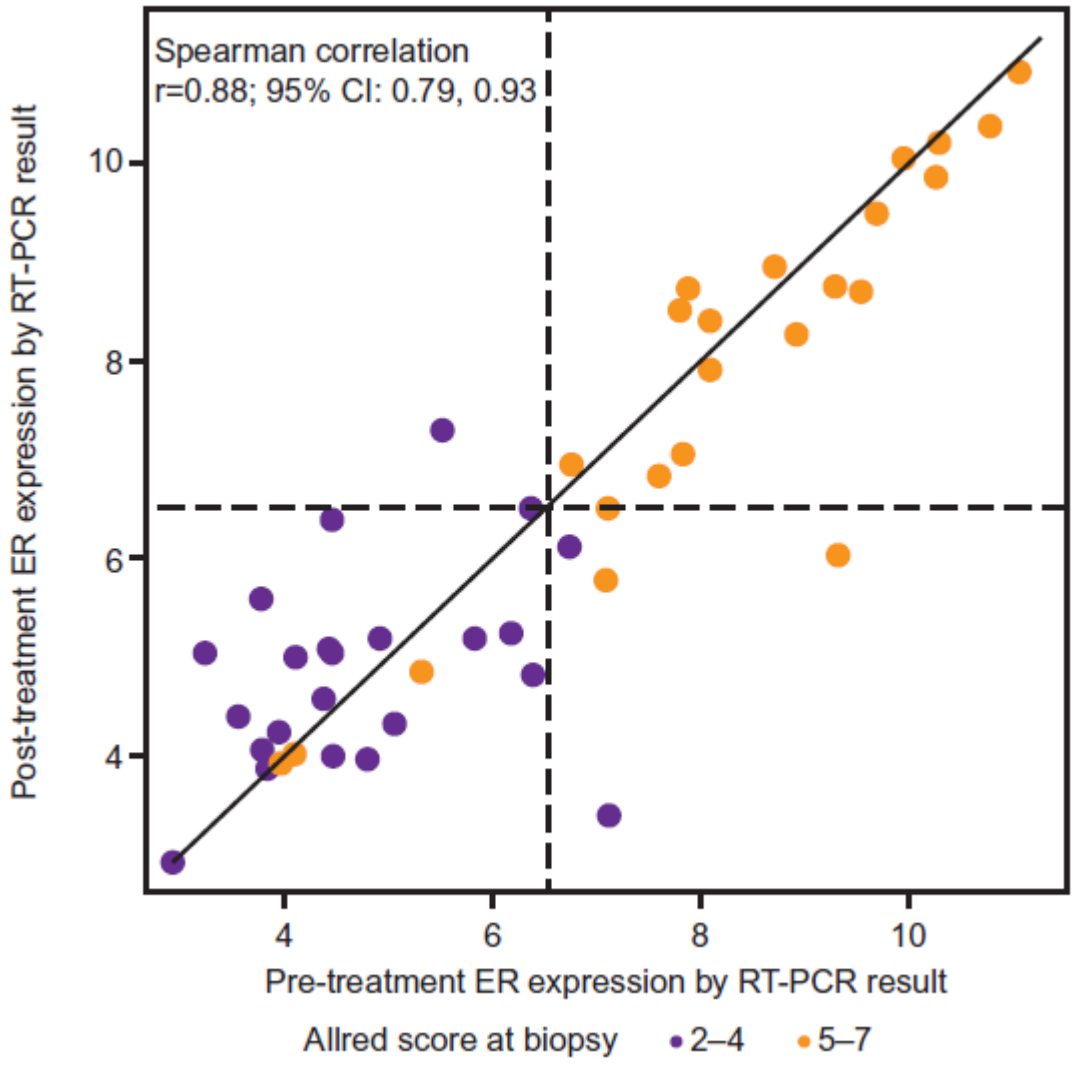
ER estrogen receptor, *IHC* immunohistochemistry, *RT-PCR* reverse-transcriptase polymerase chain reaction

Fig. S2 Scatterplot of Recurrence Score results by continuous ER (IHC or RT-PCR); IHC (a), RT-PCT (b)



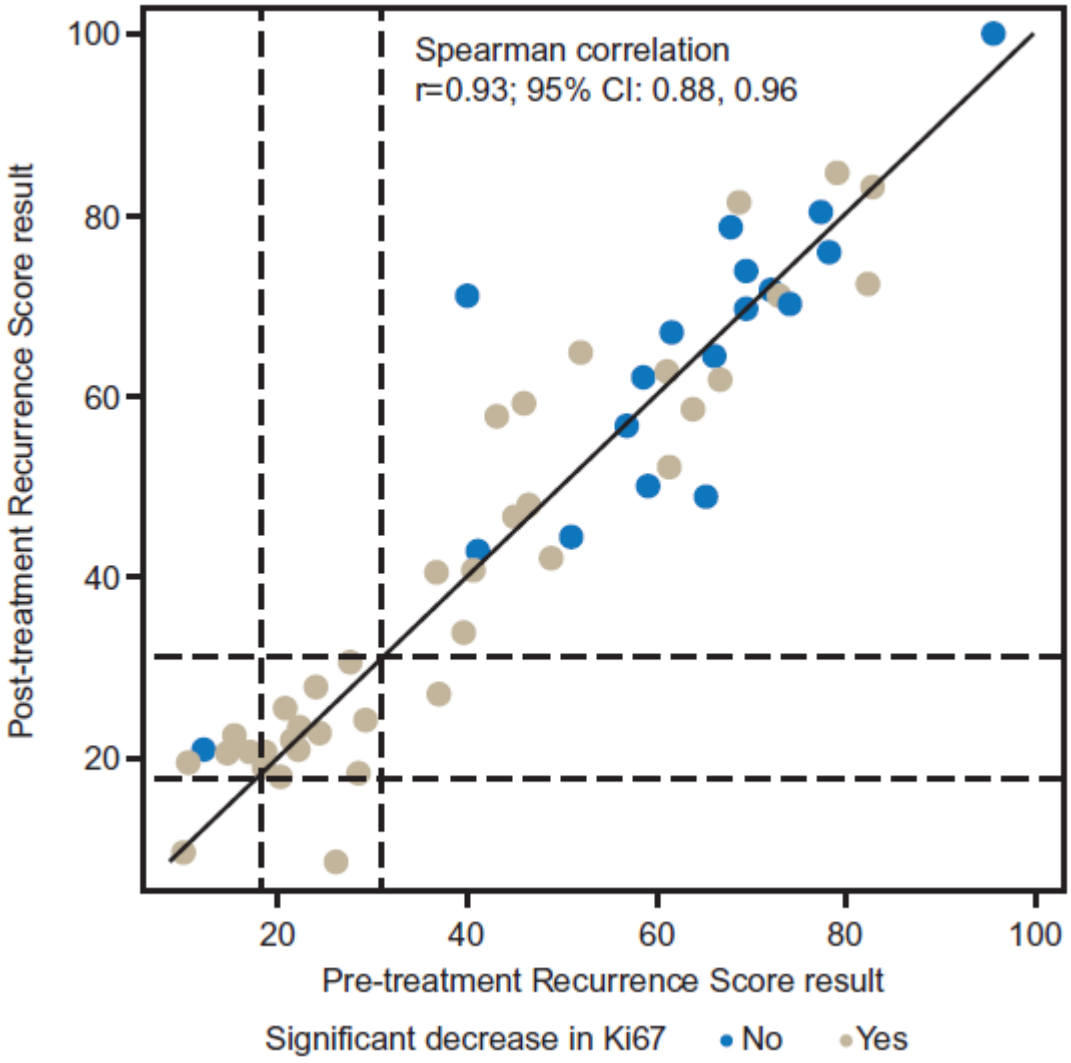
ER estrogen receptor, *CI* confidence interval, *IHC* immunohistochemistry, *RT-PCR* reverse-transcriptase polymerase chain reaction

Fig. S3 Change in ER expression (RT-PCR)



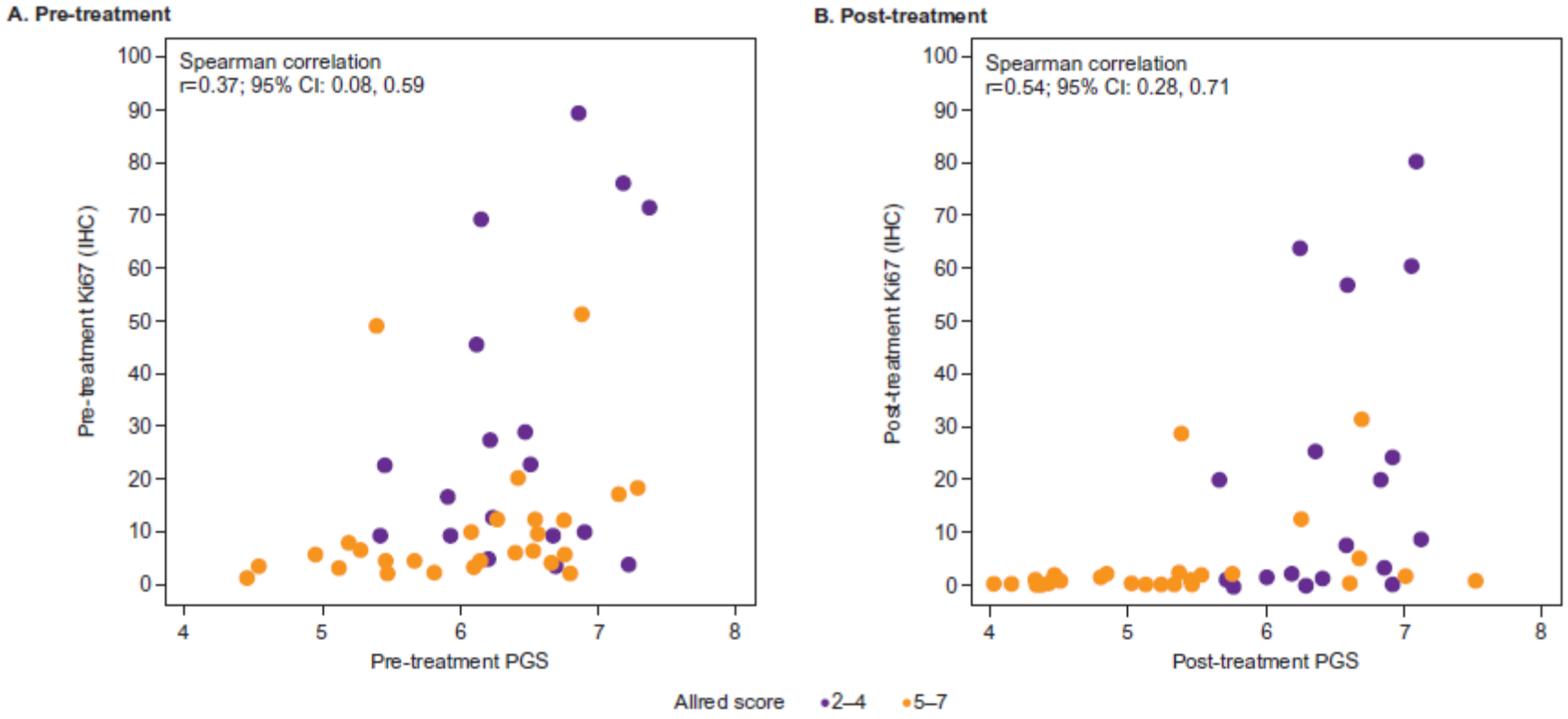
ER estrogen receptor, *RT-PCR* reverse-transcriptase polymerase chain reaction

Fig. S4. Changes in Recurrence Score results relative to Ki67 change after AI pulse (IHC)



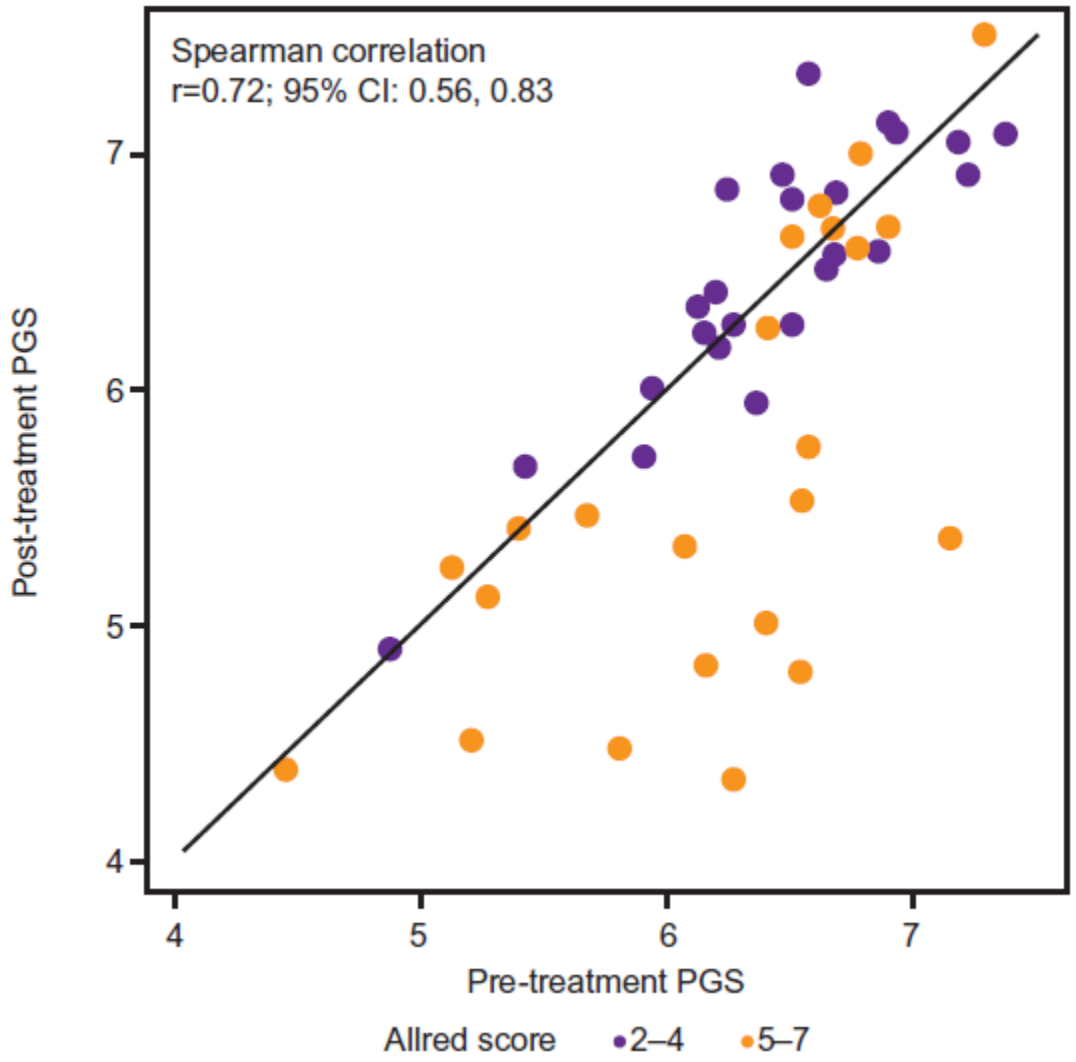
AI aromatase inhibitor, *CI* confidence interval, *IHC* immunohistochemistry

Fig. S5 Comparison of Ki67 vs. PGS pre- (a) and post-treatment (b) with AI



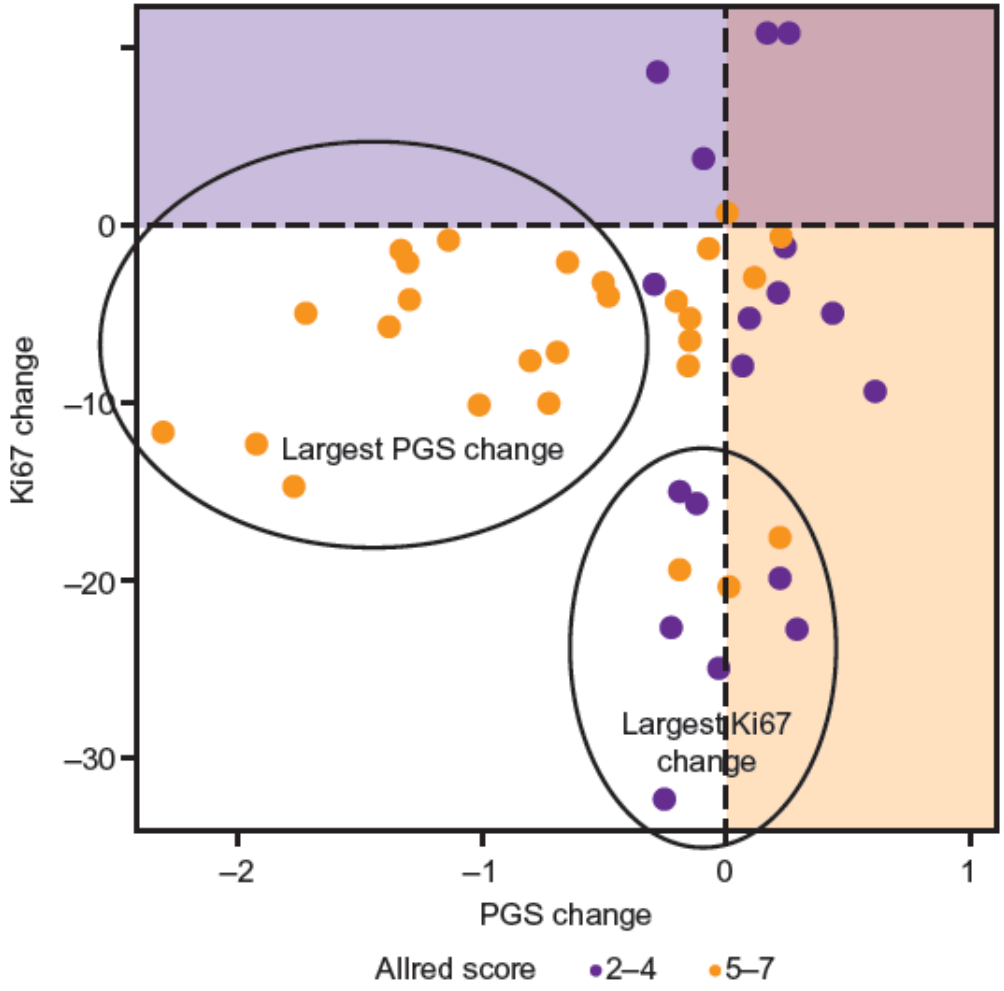
AI, aromatase inhibitor, *PGS* proliferation gene group score

Fig. S6 PGS changes post-treatment with AI



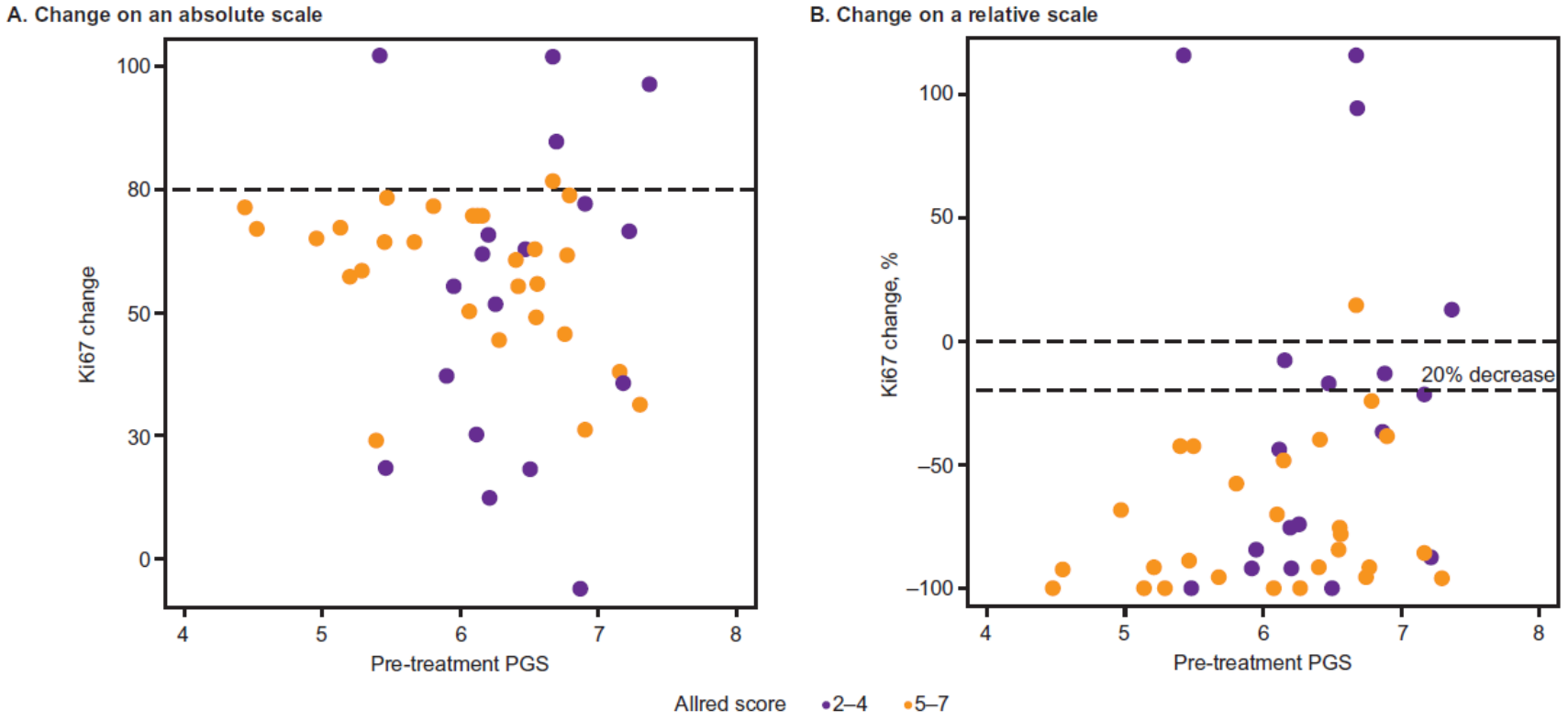
AI aromatase inhibitor, *PGS* proliferation gene group score

Fig. S7 Comparison of change in Ki67 expression versus PGS following 2 weeks of aromatase inhibitor therapy. Tumors with no decrease in Ki67 are shown in the purple box, while tumors with no decrease in PGS are shown in the orange box



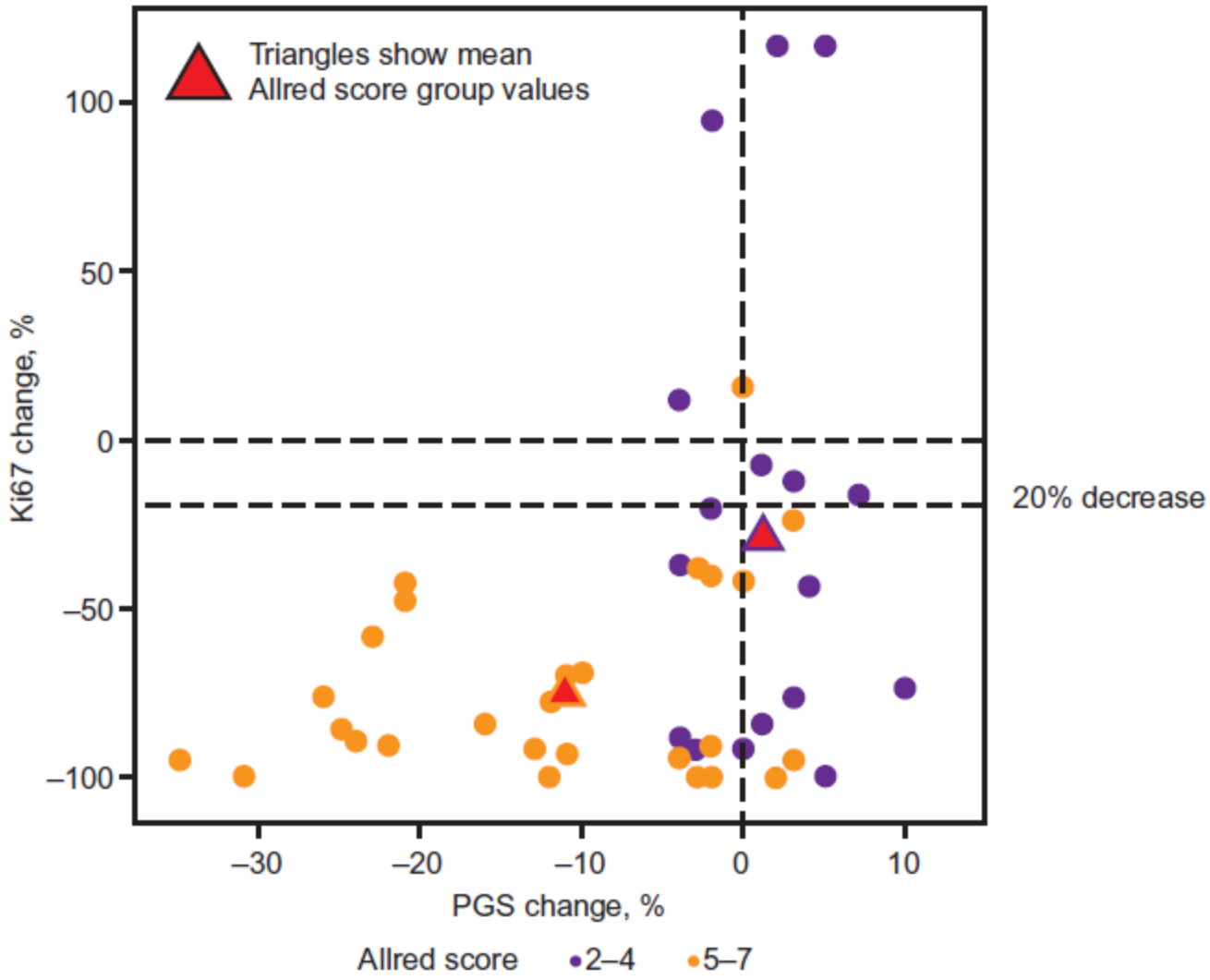
PGS proliferation gene group score

Fig. S8 Change in Ki67 with pre-treatment PGS; change on an absolute scale (a) and change on a relative scale (b)



PGS proliferation gene group score

Fig. S9 Relative reductions in Allred score



PGS proliferation gene group score, *ER* estrogen receptor